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Inhibition of NF-κB induces a switch from CD95L-dependent to CD95L-independent and JNK-mediated apoptosis in T cells

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ABSTRACT

NF-κB is a crucial transcription factor regulating apoptosis sensitivity and resistance. It has been shown that inhibition of NF-κB in T lymphocytes leads to sensitization towards apoptosis. The underlying molecular mechanism is not entirely understood. Therefore, we investigated T cell receptor (TCR) stimulated apoptosis in T cells in which NF-κB activity is blocked by an inhibitor or IκBα overexpression. We show that enhanced apoptosis upon TCR stimulation is caspase- and JNK-dependent, but independent of the CD95/CD95L system. Generation of reactive oxygen species (ROS) induced sustained JNK phosphorylation by inactivation of MAP kinase phosphatase 7 (MKP7). Sustained JNK activation causes upregulation of the pro-apototic protein BIM. Thus, inhibition of NF-κB causes a switch from classical activation-induced cell death (AICD) to CD95L-independent apoptosis.

1. Introduction

Control of T lymphocyte function and proliferation is crucial for the homeostatic balance of the immune system. Disturbance of cellular homeostasis, either by uncontrolled proliferation or excessive loss of lymphocytes, has substantial effects in disease states such as cancer, autoimmunity and AIDS. One process by which the immune system is kept in balance is TCR-mediated apoptosis of pre-activated lymphocytes (AICD) [1]. Triggering of TCR induces expression of the CD95L which is secreted and binds to CD95, thus inducing a CD95L-dependent apoptosis [1,2]. An important molecule involved in regulation of lymphocyte fate and execution of immune functions is the transcription factor nuclear factor kappa B (NF-κB). NF-κB determines decisions of cells between life and death by promoting survival through induction of NF-κB target genes. The products of these target genes regulate apoptosis in both normal and malignant cells [3]. The NF-κB pathway is activated by the TCR stimulation which induces expression of anti-apoptotic NF-κB target genes. These genes include c-FLIP, Bcl-2, Bcl-X_L, and IAP/XIAP [4]. In conclusion, inhibition of the NF-κB pathway and simultaneous TCR stimulation increase cell death in peripheral T cells of humans and mice [5,6]. Therefore, targeting the NF-κB pathway might yield new therapeutic strategies for clinical treatment of haematopoietic malignancies. Recently, we have shown that inhibition of the NF-κB pathway induces cell death in cells from cutaneous T cell lymphoma (CTCL) patients by deregulation of iron homeostasis [7].

It was shown that NF-κB is involved in the regulation of reactive oxygen species (ROS) by mediating the upregulation of anti-oxidative proteins including manganese superoxide dismutase (MnSOD, SOD2) and ferritin heavy chain (FHC) in fibroblasts upon TNFα stimulation [8,9]. $P65^{-/-}$ fibroblasts showed increased ROS levels leading to increased cell death upon TNFα stimulation [8,9]. Anti-oxidant agents reduced TNFα-mediated cell death in mouse embryonic fibroblasts.

We have shown that TCR stimulation induces an oxidative signal crucial for induction of CD95/CD95L dependent AICD [10,11]. NF-κB regulates the expression of several anti-oxidative proteins [8,9]. Therefore, we were interested in the mechanisms that are involved in TCR-induced apoptosis in T cells with impaired NF-κB activation.

Here we show that inhibition of the NF-κB pathway by a chemical compound or overexpression of IκBα in the Jurkat T cell line J16-145 (named Jurkat-IκBα) strongly increased apoptosis upon TCR or PMA/ionomycin stimulation. Cell death was independent of the CD95- (Fas, APO-1)/CD95L system but caspase-dependent. Interestingly, we observed that apoptosis induction of Jurkat-IκBα cells requires ROS generation. Investigating MAPK signaling, we observed a ROS-dependent activation of the pro-apoptotic JNK pathway. Further, siRNA-mediated knock-down of JNK abrogated

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apoptosis in Jurkat-IκBα cells showing that JNK activation is essential for induction of cell death. Sustained JNK activation was caused by ROS-inactivated MAPK phosphatase 7 (MKP7). We show that overexpression of MKP7 blocked apoptosis induction. In summary, this is the first evidence that inhibition of NF-κB causes a shift from classical CD95L-dependent TCR-induced AICD towards a CD95-independent apoptosis.

2. Materials and methods

2.1. Chemicals and antibodies

N-Acetyl-L-cysteine (NAC) was purchased from Sigma–Aldrich. Pan-caspase inhibitor zVAD was obtained from R&D Systems. Dichlorodihydrofluorescein-diacetate (H_2DCFDA) was obtained from Molecular Probes. The neutralizing anti-CD95L antibody NOK1 was obtained from BD Pharmingen. AnnexinV-FITC antibody was obtained from Immunotools. AnnexinV-APC antibody and 7-amino-actinomycin were obtained from BD Bioscience Pharmingen. HA-tagged JNK was kindly provided by Peter Angel, DKFZ, Heidelberg. Dimethylfumarate and monomethylfumarate were purchased by Sigma–Aldrich. Antibodies are described in Supplementary materials and methods.

2.2. Cell culture

Jurkat J16-145 cells [11] were cultured in Roswell Park Memorial Institute (RPMI) medium supplemented with 10% FCS and 1 mM L-glutamine.

2.3. Isolation of human lymphocytes

Human peripheral T cells were purified as described previously [7]. For activation, resting T cells were cultured at a concentration of 2×10^6 cells/ml with 1 μg/ml phytohemagglutinin for 16 h. Next, T cells were cultured in RPMI 1640 supplemented with 10% FCS and 25 U/ml interleukin 2 for 6 days. All experiments were performed with T cells isolated from at least three different donors.

2.4. Western blot analysis

1×10^6 J16-145 cells were lysed for 10 min in ice-cold RIPA lysis buffer (50 mM Tris–HCl, pH 8.0, 120 mM NaCl, 1% NP-40, 0.5% Na-Desoxycholat, 0.1% SDS, 2 mM EDTA, 25 mM NaF, 0.2 mM $NaVO_4$, 1 mM DTT, and complete protease inhibitor cocktail from Roche). Proteins were separated by SDS–PAGE and blotted onto nitrocellulose membrane (Amersham Biosciences, Little Chalfon, UK).

2.5. Phosphatase assays

To obtain phosphorylated HA-tagged JNK, HA-JNK plasmid was transiently transfected into Jurkat J16-145 cells. After 24 h, cells were stimulated with 10 ng/ml PMA and 10 μM ionomycin for 1 h and lysed with RIPA buffer without DTT. Phosphorylated HA-JNK was immunoprecipitated with Protein A beads and p-HA-JNK was eluted with elution buffer. Meanwhile, Jurkat-IκBα cells were either left untreated or pre-incubated with 20 mM *N*-acetyl cysteine for 30 min. Then, cells were stimulated with 10 ng/ml PMA and 10 μM ionomycin for 4 h. The cells were lysed in ice-cold RIPA buffer without phosphatase inhibitors. The lysate was depleted of JNK1 and JNK2 by specific antibodies and it was incubated with p-HA-JNK for 0, 5, 15, and 30 min at 37 °C. Proteins were blotted and analyzed by phospho-JNK antibodies.

2.6. ROS assays

ROS levels were assayed as described recently [12]. Cells were stained with the oxidation-sensitive dye H_2DCFDA (5 μM) for 30 min. Then, the cells were treated with 10 ng PMA or plate-bound anti-CD3 for 1 h. Treatment was terminated by addition of ice-cold PBS and ROS generation was determined by flow cytometry. The increase in fluorescence of treated vs. untreated samples is shown (increase in MFI (%) = $[(MFI_{stimulated} - MFI_{unstimulated})/MFI_{unstimulated}] \times 100$).

2.7. siRNA transfection and knock-down

J16-145 cells were transfected by lipofection (HiPerfect; Qiagen) with Allstars Negative Control siRNA (Qiagen), siRNA oligonucleotides specific for ERK2 (Qiagen), JNK1 (MAPK8, SI00300783, Qiagen), JNK2 (MAPK9, SI02222913, Qiagen). 1×10^5 Jurkat J16-145 cells in 90 μl FCS free media were incubated with 7.5 μl of HiPerfect together with indicated amounts of siRNA oligonucleotides according to the manufacturer's instructions. After 6 h cells were resuspended in 500 μl FCS containing media. For siRNA transfection of MKP and BIM the Amaxa protocol for Jurkat cells was used. BIM1 siRNA: 5′-ggaucgcccaagaguugcggctt-3′. BIM2 siRNA 5′-ggccuauucucagaggauuautt-3′. MKP7 (DUSP16, SI85692529, Qiagen). MKP7 (DUSP16, #4390824, Ambion). MKP1 (DUSP1, SI00374801, Qiagen). MKP3 (DUSP6, SI0030324, Qiagen).

2.8. Cell death assays

For cell death induction, Jurkat cells were stimulated with 30 μg/ml plate-bound anti-CD3 and apoptosis or with 10 ng/ml PMA and 10 μM ionomycin for the indicated time points. Cell death was assessed by forward-to-side-scatter (FSC/SSC) profile [13]. Specific cell death was calculated by using the following equation: specific cell death (%) = (% experimental cell death − % spontaneous cell death)/(100% − % spontaneous cell death) × 100.

3. Results

3.1. Inhibition of the NF-κB pathway induces enhanced AICD

To investigate the role of the NF-κB pathway in TCR-mediated apoptosis, we incubated primary pre-activated T cells and J16-145 T cells with dimethylfumarate (DMF) – an NF-κB inhibitor (Supplementary Fig. 1A) [14]. Treatment with DMF caused an increase in apoptosis in primary T cells as well as in J16-145 cells (Fig. 1A and B) confirming previous data showing enhanced T cell death upon inhibition of NF-κB [5,6]. As expected, apoptosis in untreated cells was dependent on caspases and CD95L (Fig. 1A and B) [15]. Interestingly, we observed that cell death upon DMF treatment was dependent on caspases, however, independent of CD95L. This indicates that the mechanism of TCR-induced cell death after inhibition of the NF-κB pathway is distinct from the pathway with intact NF-κB signaling. To further explore the molecular mechanism of this increased cell death, we established Jurkat J16-145 cells stably overexpressing IκBα (Jurkat-IκBα cells) (Fig. 1C). The capability of Jurkat-IκBα cells to activate the NF-κB pathway was strongly impaired (Fig. 1D). Next, we analyzed whether apoptosis levels were altered in Jurkat-IκBα cells compared to vector control cells upon TCR or PMA/ionomycin stimulation. Indeed, inhibition of NF-κB in Jurkat cells resulted in a strongly enhanced cell death (Fig. 1E and F). This cell death was apoptotic as revealed by DNA fragmentation (Supplementary Fig. 1B) and Annexin-V and 7-AAD staining in DMF treated cells (Supplementary Fig. 1C and D). In conclusion, apoptosis induced

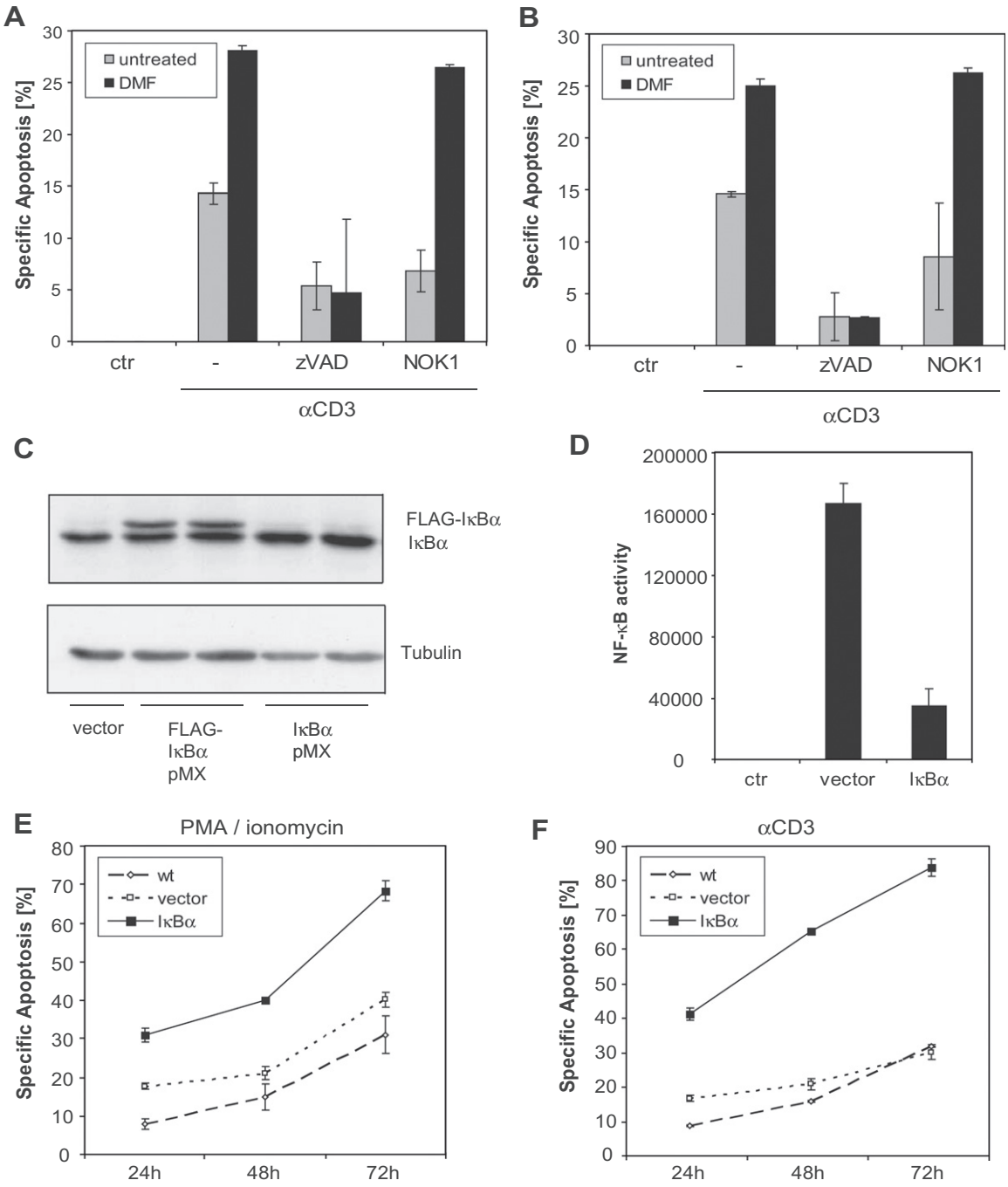

Fig. 1. Inhibition of the NF-κB pathway enhances TCR-induced apoptosis. (A) Pre-activated T cells (day 6) were either left untreated or treated with 10 μg/ml DMF. Cells were stimulated with anti-CD3 and apoptosis was determined at 48 h of culture. Where indicated T cells were pre-incubated with 20 μM zVAD or 5 ng/ml with NOK1. (B) Same as in (A), but Jurkat J16-145 cells were used. (C) Stable overexpression of IκBα and of Flag-tagged IκBα was verified by Western blot. (D) Vector control and Jurkat-IκBα cells were transfected with a plasmid encoding luciferase under transcriptional control of four NF-κB binding sites. Cells were stimulated with 10 ng/ml PMA for 8 h, then the luciferase activity was measured. (E) Wt, vector control, and Jurkat-IκBα cells were stimulated with PMA/ionomycin for the indicated time points. Apoptosis was determined by FACS. (F) Wt, vector control, and Jurkat-IκBα cells were stimulated with anti-CD3 antibody. Apoptosis was determined by FACS.

by TCR or PMA/ionomycin stimulation is increased in Jurkat-IκBα cells.

3.2. Apoptosis of Jurkat-IκBα cells is CD95L-independent but ROS-dependent

Previous work showed that ROS, as a second messenger, is required for induction of apoptosis in primary TCR stimulated T cells [11,12]. Thus, we were interested whether ROS is involved in enhanced apoptosis of Jurkat-IκBα cells. We observed that application of anti-oxidants such as NAC completely abrogated cell death in vector control and in Jurkat-IκBα cells after TCR stimulation (Fig. 2A).

In T cells, ROS enhance NF-κB and AP1 activation which finally induce CD95L expression and CD95-dependent apoptosis [12]. To investigate whether the CD95/CD95L system is involved in enhanced apoptosis after NF-κB inhibition, we co-incubated cells with a CD95L neutralizing antibody (NOK1). As expected,

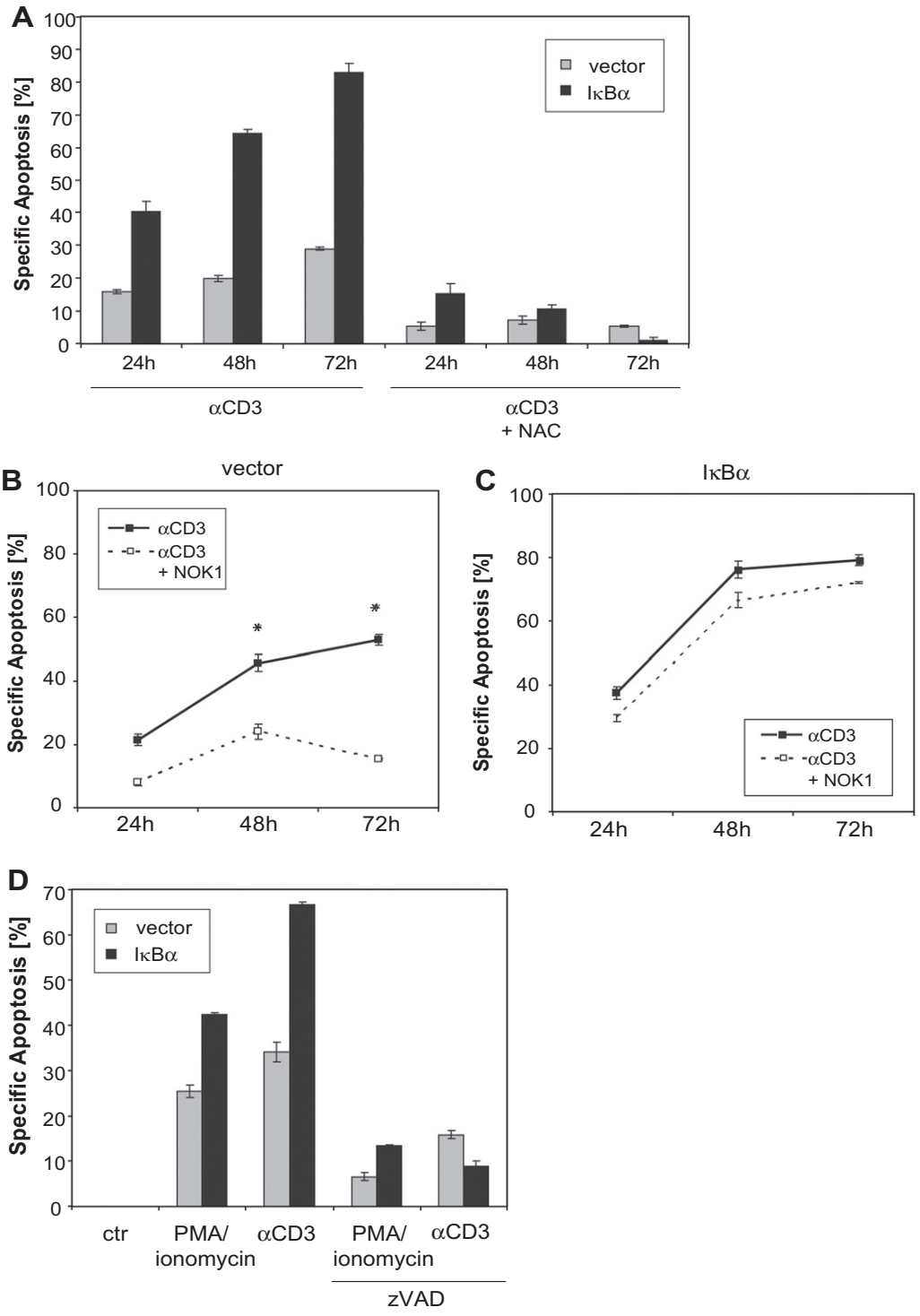

Fig. 2. Apoptosis in Jurkat-IκBα cells is dependent on ROS. (A) Vector control and Jurkat-IκBα cells were either left untreated or pre-incubated with 20 mM NAC for 30 min. Then, cells were stimulated with anti-CD3 antibody for the indicated time points. Apoptosis was determined by FACS. (B) Vector control cells were either left untreated or pre-incubated with 5 ng/ml NOK1. Then, cells were stimulated with anti-CD3 antibody and apoptosis was determined. Results are representative for three individual experiments. * $P < 0.05$ according to Student's *t*-test. (C) Jurkat-IκBα cells were either left untreated or pre-incubated with NOK1. Then, cells were stimulated with plate-bound anti-CD3 antibody and apoptosis was determined by FACS. Results are representative for three individual experiments. (D) Vector control and Jurkat-IκBα cells were either left untreated or pre-incubated with 20 μM zVAD for 15 min. Then, cells were stimulated with PMA/ionomycin or with 30 μg/ml plate-bound anti-CD3 antibody for 48 h. Apoptosis was determined by FACS.

we observed that apoptosis of vector control cells was dependent on CD95L (Fig. 2B), but apoptosis of Jurkat-IκBα cells was independent of the CD95 system, since NOK1 did not interfere with apoptosis induction corroborating our results in primary T cells (Fig. 2C). In addition, we detected that cell death in Jurkat-IκBα cells was blocked by the pan-caspase inhibitor z-VAD (Fig. 2D). Thus, we demonstrated that apoptosis in Jurkat-IκBα cells does not require CD95/CD95L but is strongly dependent on ROS generation and caspase activation.

3.3. Jurkat-IκBα cells show increased ROS generation

Since ROS play an important role in apoptosis, we asked whether expression of anti-oxidative enzymes is altered in Jurkat-IκBα cells. MnSOD and FHC are typical NF-κB targets of major importance for the anti-oxidative defence [7–9]. Therefore, we determined mRNA expression levels of MnSOD and FHC by quantitative RT-PCR. MnSOD mRNA levels were upregulated by about two-fold in vector control cells upon stimulation (Fig. 3A). However, Jurkat-IκBα cells did not show an upregulation of MnSOD upon TCR triggering or

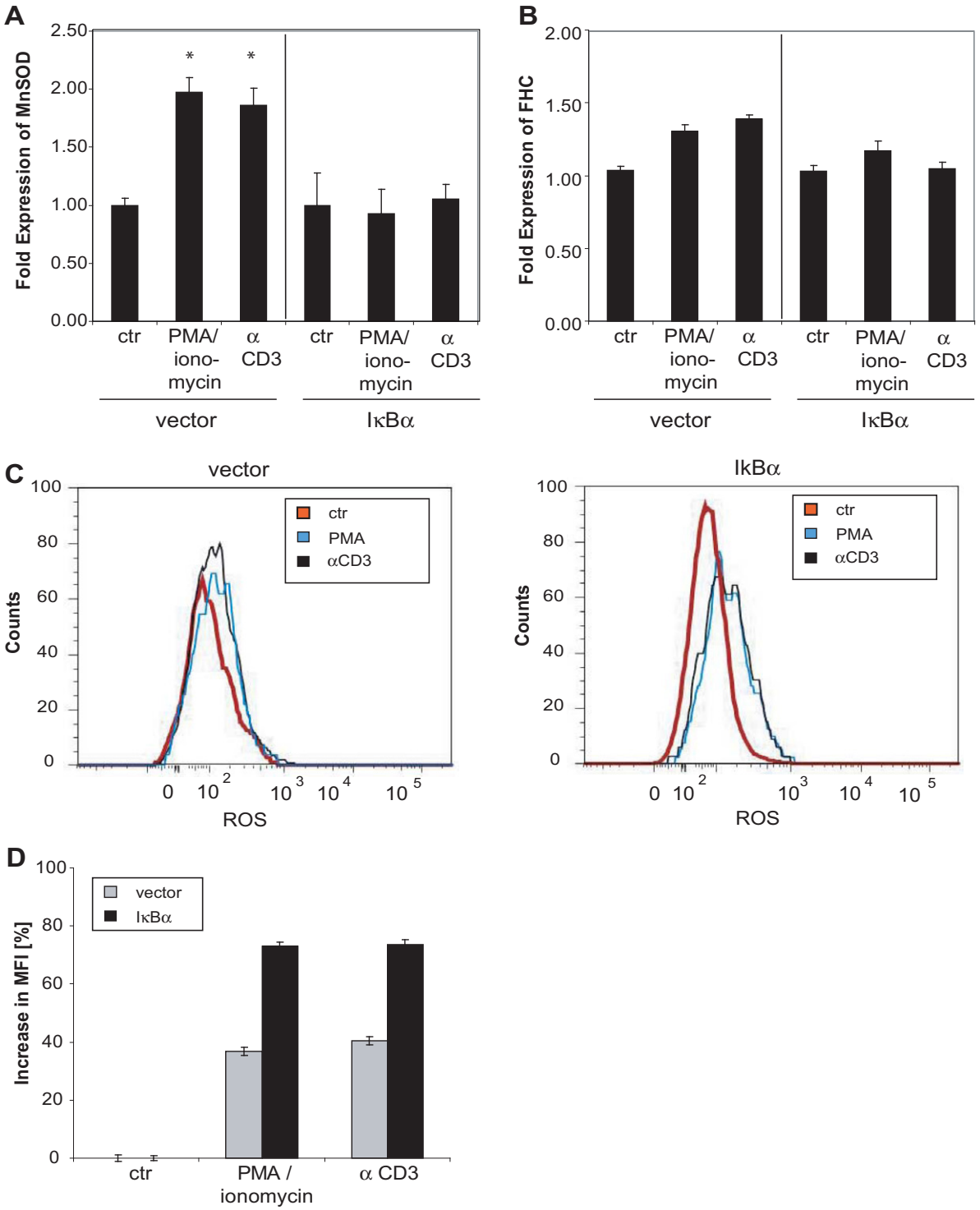

Fig. 3. Jurkat-IκBα cells show reduced expression of anti-oxidative enzymes and higher ROS levels. (A) Vector control and Jurkat-IκBα cells were stimulated with PMA/ionomycin or with anti-CD3 for 3 h. Subsequently, cells were lysed and RNA was isolated. Expression levels of MnSOD were assessed by quantitative real-time PCR. Results are representative for three individual experiments. * $P < 0.05$ according to Student's *t*-test. (B) Vector control and Jurkat-IκBα cells were stimulated with 10 ng/ml PMA and 10 μM ionomycin or with 30 μg/ml plate-bound anti-CD3 for 5 h. Expression levels of FHC were assessed by quantitative real-time PCR. (C) Vector control cells (left panel) or Jurkat-IκBα cells (right panel) were pre-incubated with H_2DCFDA for 10 min, followed by stimulation with either 10 ng/ml PMA and 10 μM ionomycin or with 30 μg/ml plate-bound anti-CD3 for 1 h. Then, fluorescence of H_2DCFDA was detected by FACS. (D) Same as in (C), but results are shown as mean fluorescence intensity. Each bar represents a biological triplicate.

PMA/ionomycin stimulation (Fig. 3A). A similar result, although not as pronounced, was obtained for FHC. FHC expression was induced in vector control cells upon stimulation but not in Jurkat-IκBα cells (Fig. 3B). The expression levels of other putative NF-κB target genes including Bcl-2, Bcl-X_L, c-FLIP, and XIAP were not altered in Jurkat-IκBα cells (Supplementary Fig. 2A).

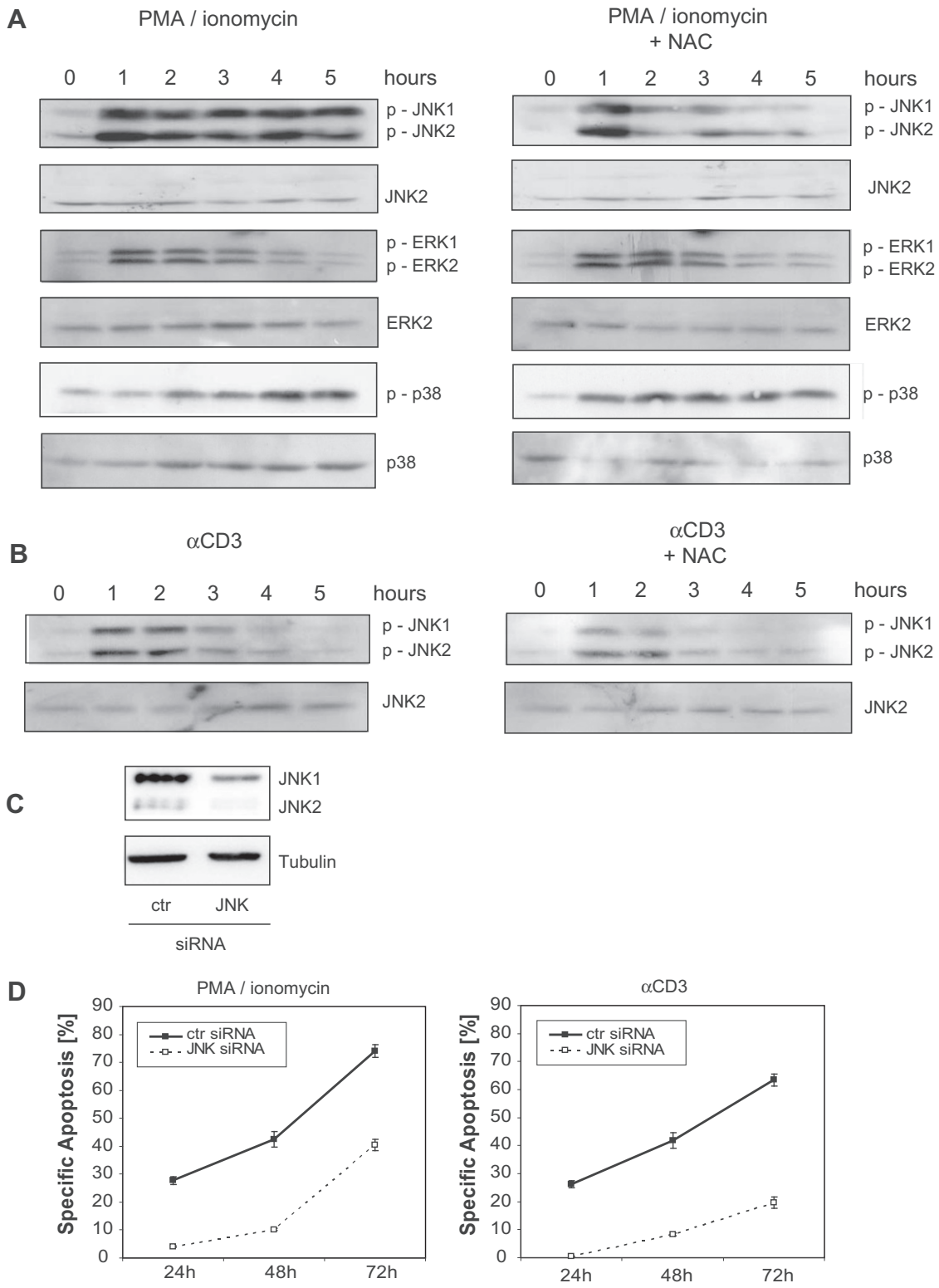

Fig. 4. Inhibition of NF-κB results in prolonged JNK activation which is required for apoptosis. (A) Jurkat-IκBα cells were either left untreated or pre-incubated with 20 mM NAC. Then, cells were stimulated with PMA/ionomycin. Phosphorylation status of JNK1/JNK2, ERK1/ERK2, and p38 was analyzed. Results are representative for five individual experiments. (B) Jurkat-IκBα cells were either left untreated or pre-incubated with 20 mM NAC. Then, cells were stimulated with anti-CD3 antibody for the indicated time points. Cells were lysed and the phosphorylation status of JNK1/2 was assessed. (C) siRNA-mediated knock-down of JNK1 was verified by Western blot. Jurkat-IκBα cells were treated with control siRNA, ERK siRNA and with JNK siRNA for 48 h. (D) Jurkat-IκBα cells were treated with control siRNA or JNK siRNA for 48 h, then stimulated with either 10 ng/ml PMA and 10 μM ionomycin (left panel) or with anti-CD3 antibody (right panel). Apoptosis was determined by FACS.

Next, we investigated whether the diminished expression of anti-oxidative enzymes translates into alteration of ROS levels. We did not observe altered basal ROS levels in the two cell lines investigated. However, we found that generation of ROS after stimulation was significantly enhanced in Jurkat-IκBα cells compared to control cells (Fig. 3C and D). This supports our finding that

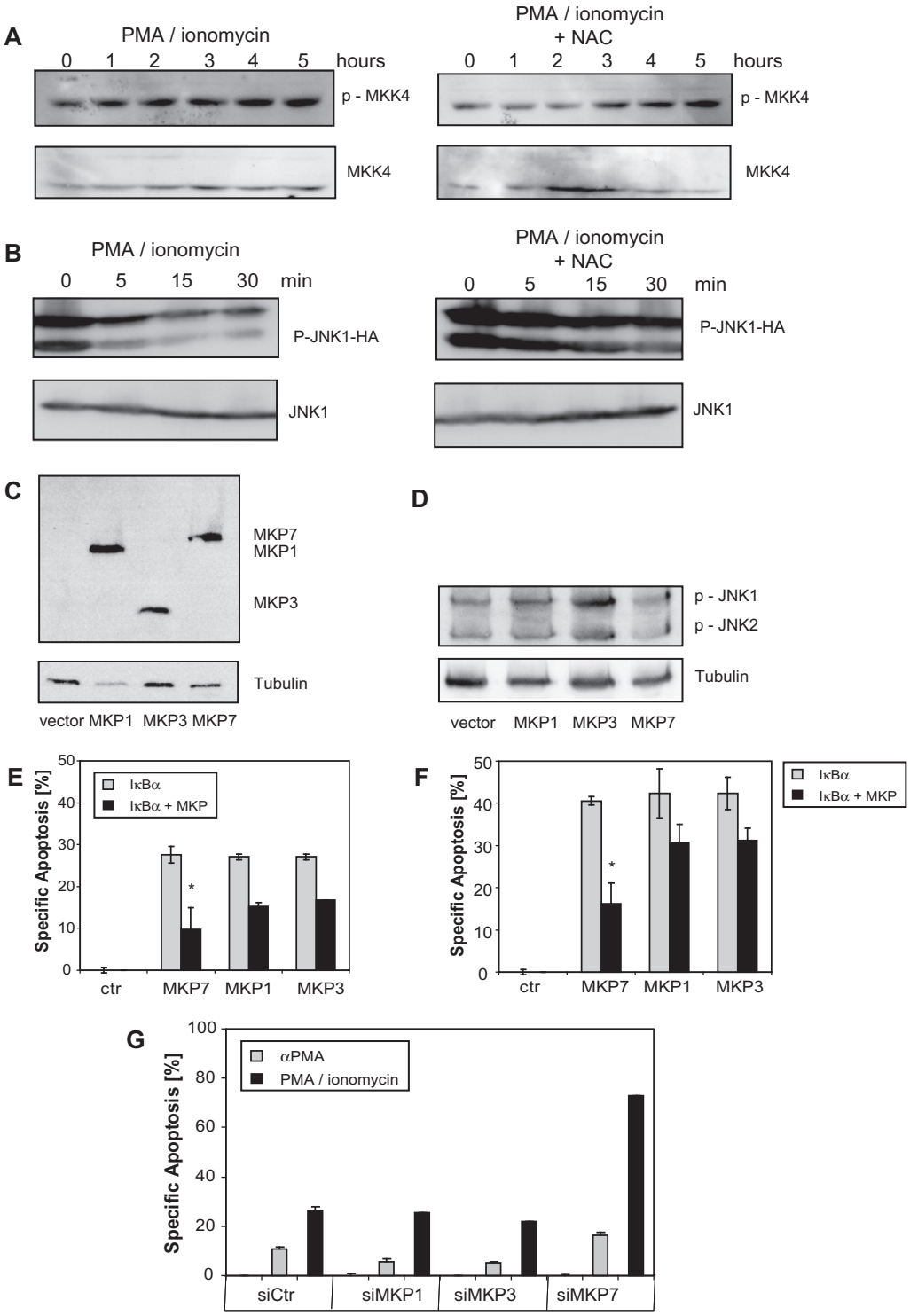

Fig. 5. Prolonged JNK activation by inactivation of phosphatases. (A) Jurkat-IκBα cells were either left untreated or pre-incubated with 20 mM NAC. Then, cells were stimulated with PMA/ionomycin for the indicated time points. Cells were lysed and the phosphorylation status of MKK4 was assessed. (B) Jurkat-IκBα cells were either left untreated or pre-incubated with 20 mM *N*-acetyl cysteine. Then, cells were stimulated with PMA/ionomycin for 4 h. Cells were lysed and lysates were incubated for the indicated time points with phospho-JNK1-HA. Lysates and phospho-JNK1-HA was subjected to Western blot. (C) Overexpression of MKP1, MKP3, and MKP7 was verified by Western blot in 293T cells. (D) A control vector or MKP1, MKP3, or MKP7 were overexpressed in Jurkat cells and stimulated with PMA/ionomycin for 2 h. (E and F) Jurkat-IκBα cells were transiently transfected with a control vector or with a vector encoding MKP1, MKP3, or MKP7. Control vector or MKP transfected cells were sorted according to GFP co-expression. Then, sorted cells were stimulated with PMA/ionomycin or with anti-CD3 antibody for the indicated time points. Apoptosis was determined by FACS. (G) Control siRNA or siRNAs against MKP1, MKP3, or MKP7 were transfected in Jurkat cells. After 48 h, Jurkat cells were either stimulated with anti-CD3 antibody or with PMA/ionomycin for 48 h. Results are representative for two individual experiments.

Jurkat-IκBα cells have a reduced capability of anti-oxidative defence.

3.4. Inhibition of NF-κB results in enhanced JNK activation which is required for apoptosis

TCR stimulation results in activation of MAPK pathways. Previous data have shown that ROS regulates the activity of MAPKs after TCR stimulation only to a marginal extent [16]. We detected that JNK phosphorylation peaks after 2–3 h after stimulation and declines thereafter (Supplementary Fig. 2B). The antioxidant NAC slightly increased activation and phosphorylation levels of ERK in Jurkat cells (Supplementary Fig. 2B and C). On the contrary, the phosphorylation status of stress response pathways including JNK and p38 MAPKs were only marginally reduced by NAC (Supplementary Fig. 2B and C). In addition, we explored the activation of JNK, p38 MAPK, and ERK in Jurkat-IκBα cells. Compared to Jurkat J16-145 cells in which JNK activation was terminated at 2 h, we observed sustained JNK phosphorylation up to 5 h in Jurkat-IκBα cells after PMA/ionomycin stimulation (Fig. 4A, left panel). To check whether sustained JNK activation was mediated by ROS we co-incubated cells with NAC before stimulation. The addition of NAC resulted in potent abrogation of JNK phosphorylation after 2 h (Fig. 4A, right panel). The activation status of ERK and p38 was similar in vector control and Jurkat-IκBα cells and was not significantly altered by addition of NAC (Fig. 4A). In addition, JNK phosphorylation by TCR stimulation in Jurkat-IκBα cells was also dependent on ROS as it was blocked by NAC (Fig. 4B). Thus, enhanced ROS generation in stimulated Jurkat-IκBα cells induces prolonged JNK but not ERK or p38 MAPK activation. To study whether JNK is required for execution of apoptosis we targeted JNK expression by siRNA. Treatment of Jurkat-IκBα cells with

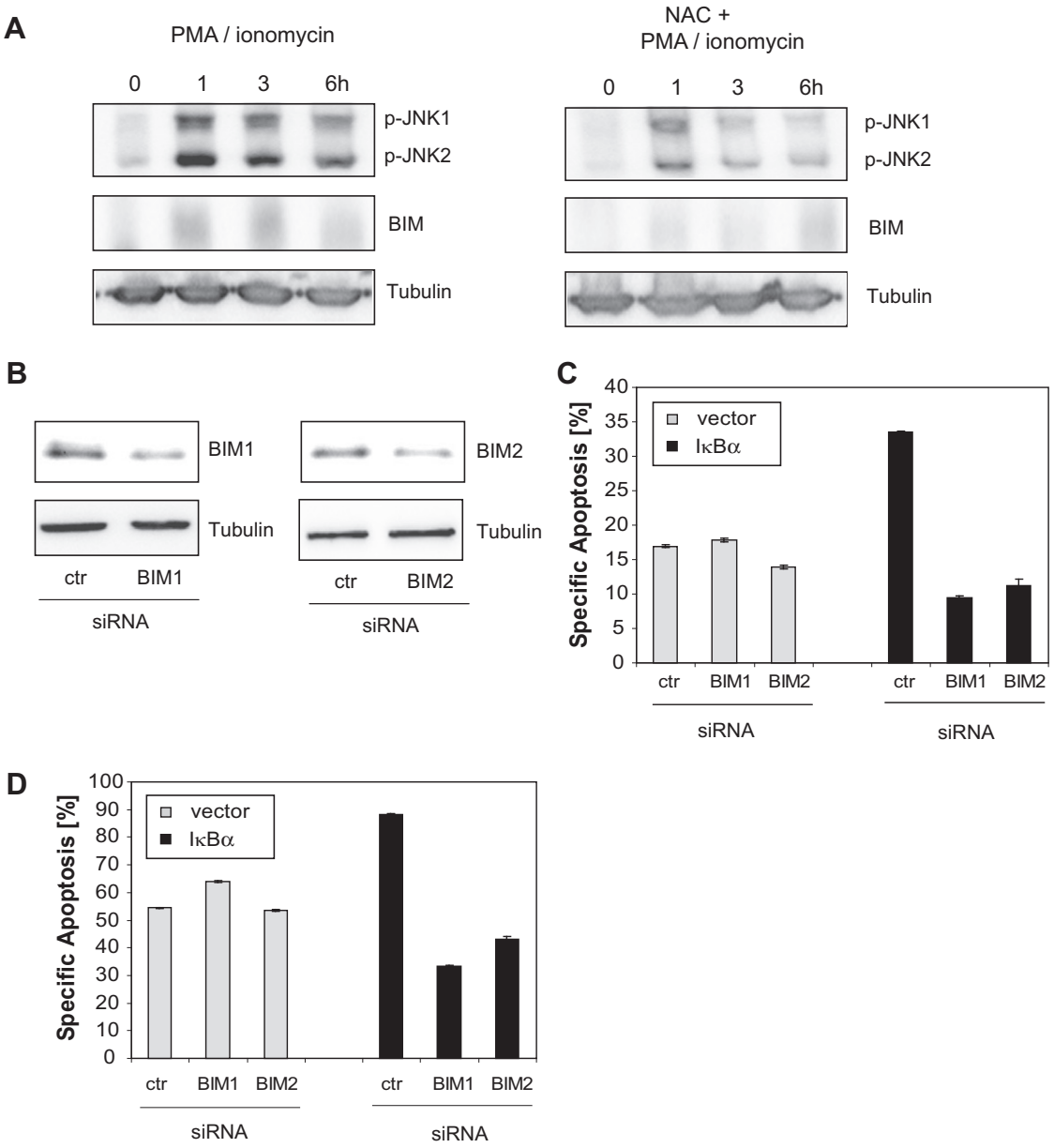

Fig. 6. BIM is important for apoptosis in Jurkat-IκBα cells. (A) Jurkat-IκBα cells were either left untreated or pre-incubated with 20 mM NAC. Then, cells were stimulated with PMA/ionomycin for the indicated time points. Cells were lysed and the phosphorylation status of JNK1/JNK2, BIM, and tubulin was assessed by specific antibodies. (B) Knockdown of BIM by two specific siRNAs was verified by Western blot. (C and D) Vector control cells and Jurkat-IκBα cells were treated with control siRNA, or two siRNAs against BIM for 48 h. Then, cells were stimulated either with anti-CD3 antibody (C) or with PMA/ionomycin (D) antibody for 24 h. Apoptosis was determined by FACS.

specific siRNA against JNK1/2 induced downregulation of JNK1 and JNK2 on the protein level after 48 h compared to control siRNA treated cells (Fig. 4C). Downregulation of JNK resulted in significantly reduced apoptosis after stimulation (Fig. 4D).

3.5. Phosphatases are critically involved in apoptosis of Jurkat-IκBα cells

We wanted to investigate the molecular mechanism for prolonged JNK phosphorylation. We assumed that upstream kinases, such as MKK-4, are responsible for sustained JNK phosphorylation. However, we observed that MKK-4 phosphorylation, though activated in Jurkat-IκBα cells, was not dependent on ROS as NAC had no effect (Fig. 5A). Thus, we conclude that sustained JNK phosphorylation is not mediated by upstream kinases.

The MAPK signaling cascades are also regulated by phosphatases. Phosphatases contain a critical cysteine residue in their catalytical center which is susceptible to oxidation [17]. Oxidation of the active site cysteine abrogates phosphatase function. To examine whether phosphatase activity might be affected by ROS in Jurkat-IκBα cells, we applied a phosphatase activity assay. Phosphorylated JNK was incubated with cell extracts from stimulated Jurkat-IκBα cells left untreated or co-treated with NAC. Treatment of cells with PMA/ionomycin resulted in no or only minor phosphatase activity since phosphorylation of JNK was not altered up to 30 min of co-incubation with lysate (Fig. 5B, left panel). However, addition of NAC restored phosphatase activity and, subsequently, reduced JNK phosphorylation after 15–30 min of co-incubation with lysate (Fig. 5B, right panel). Thus, sustained JNK phosphorylation is a result of ROS-mediated inactivation of phosphatases. MKPs have rather non-redundant functions and show a high grade of specificity for the members of the MAPK family [18]. MKP1, MKP3, and MKP7 are expressed in T cells and were reported to be strongly upregulated in leukocytes after activation [18,19]. MKP7 preferentially dephosphorylates JNK rather than ERK or p38 [20,21], whereas MKP3 is specific for ERK dephosphorylation [22,23]. MKP3 and MKP7 are localized in the cytosol and MKP1 is localized in the nucleus [18]. This indicates a specific role of MKP7 dephosphorylating JNK. Overexpression of MKPs was verified by Western blot (Fig. 5C). Overexpression of MKP7 but not of MKP1 and MKP3 blocked JNK phosphorylation (Fig. 5D). Next, MKP overexpressing cells were stimulated and apoptosis was measured. Overexpression of MKP7 compared to other MKPs resulted in the strongest decrease of apoptosis (Fig. 5E and F). This shows that regulation of prolonged JNK activation by MKP7 is a crucial step for survival and apoptosis induction in Jurkat T cells. Overexpression of MKP1 and MKP3 also lead to a partial block of apoptosis (Fig. 5E and F), indicating that these phosphatases play a minor role in apoptosis sensitization by inhibition of NF-κB. To further strengthen the role of MKP7 in apoptosis regulation we specifically targeted MKP7 expression by siRNA. Knock-down of MKP7 but not of other MKPs resulted in a clear increase of apoptosis by CD3 or PMA/Ionomycin stimulation (Fig. 5G). Therefore, these results show the importance of MKP7 activity for apoptosis.

3.6. JNK induces BIM expression and BIM is important for apoptosis of Jurkat-IκBα cells

How prolonged JNK activation causes apoptosis merits further investigations. One putative mediator of CD95-independent apoptosis is BIM [24,25]. Further, it was shown that JNK can upregulate the expression of BIM [26]. Indeed, we found that prolonged JNK activation increased BIM expression whereas NAC blocked both, JNK activation and BIM expression (Fig. 6A). NAC had no effect in control Jurkat cells (data not shown). To further corroborate the role of BIM we performed a specific knock-down of BIM by siRNA (Fig. 6B). Diminished expression of BIM did not interfere with induction of apoptosis in Jurkat control cells (Fig. 6C and D) since Jurkat cells undergo apoptosis upon stimulation in a CD95L-dependent manner [27]. In contrast, apoptosis in Jurkat-IκBα cells was strongly dependent on BIM expression (Fig. 6C and D). These experiments show that JNK induces the expression of BIM an important mediator for apoptosis in Jurkat-IκBα cells.

4. Discussion

NF-κB is a crucial transcription factor involved in many physiological processes including differentiation, proliferation, and cell death. In this study, we describe the importance of NF-κB in T cells for determination of the cellular fate and mode of apoptosis upon TCR triggering. Activation of the TCR causes a plethora of signaling events involving activation of kinases such as Lck, ZAP70, and PKCθ which finally culminate in the activation of transcription factors NF-AT, NF-κB, and AP1 [28]. These and other transcription factors induce expression of the CD95L that binds CD95. Triggering of the CD95 receptor results in apoptosis [2]. Here we describe that in TCR triggered T cells in which the NF-κB pathway is blocked apoptosis does not involve CD95 engagement. Our data indicate, that apoptosis shows comparable hallmarks to TNFα-induced cell death in NF-κB deficient cells [8,9]. However, we could not observe a role for TNFα since apoptosis was not impaired by soluble TNFα-receptor Fc (Enbrel) (Supplementary Fig. 3). Here we show for the first time that a switch from classical AICD depending on the CD95/CD95L to a CD95-independent apoptosis is observed. We found that the inhibition of the NF-κB pathway impaired the upregulation of anti-oxidative enzymes usually observed after TCR stimulation (Fig. 3) [12]. Expression levels of MnSOD and FHC were not increased by TCR triggering in Jurkat-IκBα cells in which NF-κB activity is decreased. This led to a diminished anti-oxidative capacity in these cells in comparison to vector control cells with intact NF-κB activity. The diminished anti-oxidative capacity was reflected by increased ROS production in Jurkat-IκBα cells upon PMA or TCR stimulation (Fig. 3). In recent years, it has become evident that ROS can act as a second messenger, modulating and regulating several signaling pathways [29]. Transcription factors such as p53, Jun, Fos, and the p50 subunit of NF-κB can directly be oxidized altering their transcriptional activity [30]. The most prominent targets of ROS, however, are phosphatases [29]. Oxidation abolishes phosphatase activity. In our study we observed that MPK are inhibited in their function to dephosphorylate JNK in Jurkat-IκBα cells. Decreased phosphatase activity could be rescued by addition of NAC (Fig. 5). Further, we found that phosphatase inactivation caused sustained JNK phosphorylation but not inhibition of upstream kinases such as MKK4. Recent studies shed more light on subcellular localization of MKPs and possible dephosphorylation targets of these crucial regulators. Astonishingly, and in contrast to other protein phosphatases, most MKPs show a high grade of specificity for their targets. Substrate specificity assays could prove that MKP7 has a strong preference for dephosphorylating JNK and p38 over ERK [20,21], whereas MKP3 rather acts on ERK but not on JNK and p38 [22,23]. Both, MKP7 and MKP3, were reported to be strongly upregulated in leukocytes after activation [19]. Another related phosphatase, MKP1, also acts on p38 and JNK. However, MKP1 is localized in the nucleus indicating that it is not involved in cytoplasmic signaling [31–33]. This might explain why we detected that mainly MKP7 activity can inhibit apoptosis induced by TCR or PMA/ionomycin stimulation (Fig. 6). Our experiments show for the first time that MKPs are involved in regulation of TCR-triggered apoptosis. Inactivation of MKP7 by ROS, and to a minor extent of other MKPs, resulted in sustained JNK activation. Further, specific knock-down of MKP7 induced

spontaneous apoptosis but not in the case of MKP1 or MKP3 clearly showing that MKP7 plays a prominent role in apoptosis regulation. We also observed that caspases are involved in the above signaling pathway (Fig. 1) which led to the assumption that caspases are acting downstream of JNK phosphorylation. Other potential targets of JNK are Bcl-2 family members. One study showed that JNK phosphorylates Bim, thus inducing Bax-dependent mitochondrial apoptosis [34]. We observed that knockdown of BIM had some influence on apoptosis in vector cells (Fig. 6). However, the BIM knockdown did strongly affect apoptosis in Jurkat-IκBα cells, thus, demonstrating that BIM is a downstream target of sustained JNK (Fig. 6).

Based on our results, we propose that inhibition of the NF-κB pathway switches classical CD95-dependent AICD towards a mode of apoptosis in which ROS-mediated MKP inactivation and subsequent sustained JNK activity are required.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.febslet.2010.10.047.

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